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(54) Title: METHODS AND USES OF CONNECTIVE TISSUE GROWTH FACTOR AS AN INDUCTION AGENT

(57) Abstract

The present invention relates to novel methods and compositions related to the administration of connective tissue growth factor, alone or in combination with other growth factors, compositions or compounds, to induce the formation of connective tissue, including bone, cartilage, and the skin.

METHODS AND USES OF
CONNECTIVE TISSUE GROWTH FACTOR
AS AN INDUCTION AGENT

1. **STATEMENT OF RELATED CASES**

This application is related to and is a continuation-in-part application of Serial Number 08/459,717, entitled "Connective Tissue Growth Factor," filed June 2, 1995, which is a continuation-in-part application of Serial No. 08/386,680, filed on February 10, 1995, having the same title, which is a divisional application of Serial Number 08/167,628, filed December 14, 1993, now issued as U.S. Patent No. 5,408,040, which is a continuation of Serial No. 07/752,427, filed on August 30, 1991, now abandoned.

2. **FIELD OF THE INVENTION**

This invention relates generally to the field of growth factors and specifically to Connective Tissue Growth Factor (CTGF) and methods of use thereof.

3. **BACKGROUND OF THE INVENTION**

   A.   **The Role Of Growth Factors In Bone And Cartilage Formation**

   Bone And Cartilage Formation.   The formation of tissue and organs in all multicellular organisms that arise from a single fertilized egg requires the differentiation of specialized cell types from non-differentiated stem cells. As embryogenesis proceeds, more highly specialized cell types and complex structures are formed. Currently, however, little concrete information is available on the identification of

the specific factors or the mechanism of action of these
factors on skeletal or cartilage formation in vertebrate
animals, including humans.

There are two common types of bone formation in the
mammalian system: intramembranous ossification and
endochondral ossification. The formation of the bones of the
skull are an example of intramembranous ossification. There,
mesenchymal cells from the neural crest interact with the
extracellular matrix of the cranial epithelial cells and form
bone. Hall, *Amer. Sci.* , 1988, 76174-181. Mesenchymal cells
condense into small islands and differentiate into
osteoblasts and capillaries. The osteoblasts secrete a
specific type of extracellular matrix, (osteoid) which is
capable of binding calcium salts.

Endochondral ossification is the process by which the
long bones of the axial skeleton (arms and legs), and the
vertebra and ribs form. Hall, *supra*. During this process
the formation of bone occurs via a cartilaginous tissue
intermediate stage. In mammals, the long bones form from
certain mesenchymal cells in the embryonic limb buds. These
cells form chondrocytes, and secrete a cartilaginous matrix.
Other surrounding mesenchymal cells form the perichondrium
(ultimately, the periosteum). In some cases, chondrocytes
adjacent to the region where chondrocytes are proliferating
and forming differentiate into hypertrophic chondrocytes.

Hypertrophic chondrocytes produce a different type of
matrix, and alter their tissue orientation to form the
physis. The structure of the physis is arranged in multiple
cellular columns composed of zones of cellular hypertrophy,
proliferation, ossification and vascularization. Hall,
supra; Gilbert, "Transcriptional regulation of gene
expression," DEVELOPMENTAL BIOLOGY, 5th ed. Sinaur Assoc., p.
387-390 (1994). This results in a gradation of cell
transformation from chondrocytes to osteoblasts which form
the mineralized bone.

Endochondral ossification is an active, ongoing process
that occurs in mammals during the growth from infant to
adult. The differentiation of mesenchymal cells to

- 2 -

chondrocytes, their proliferation and replacement by
osteoblasts are dependent on growth factors (including the
TGF-β family), and on the mineralization of the matrix.
Tuan, 1984, *J. Exp. Zool.* (suppl.) 1:1-13 (1984); Syfestad
and Caplan, 1984, *Devel. Biol* . 104:348-386.

    With regard to connective tissue, it is felt that all
skeletal elements in mammals are derived from a single stem
cell that is capable of differentiating into the specific
cell types that compose muscle, cartilage, bone and tendon.
These cells also appear to be capable of differentiating into
adipose tissue.

        *The Relevant Art Related To Growth Factors And The
Formation Of Bone And Cartilage.* Prior to the present
invention, it was known generally that growth factors
comprise a class of secreted polypeptides that stimulate
target cells to proliferate, differentiate and organize
developing tissues. Typically, a growth factor's activity is
dependent on its ability to bind to specific receptors,
thereby stimulating a signaling event within the cell.
Examples of some well-studied growth factors include
platelet-derived growth factor (PDGF), insulin-like growth
factor (IGF-I), transforming growth factor beta family (TGF-
β), transforming growth factor alpha (TGF-α), epidermal
growth factor (EGF), and fibroblast growth factors (FGF).

        *Effect Of TGF-β On Chondrocyte Growth,
Differentiation and Cartilage Formation.* The TGF-βs play a
role in chondrogenesis. As previously reported, TGF-β1 and
TGF-β2 increase chondrogenesis in embryonic rat mesenchymal
cells (Seyedin,et al.., 1987, *J. Biol. Chem.* 262: 1946-1947),
and either isoform can induce formation of chondroblasts from
murine muscle mesenchymal cells in culture. Seyedin. et al.,
1986, *J. Biol. Chem.* 261: 5693-5695. Application of the TGF-
βs to murine embryonic prechondroid tissues increases
differentiation of mesenchymal cells, production of
proteoglycans, and replication of chondroblasts. Centrella,
et al., 1994, *Endocrine Reviews* 15:27-38; Thorp and Jakowlew,
1994, *Bone* 15: 59-64.

- 3 -

Using in-situ hybridization, decreased levels of TGF-β3
were found in the growth plates of animals with three
separate disorders where chondrocytes cease to differentiate.
Id. In organ cultures of bovine articular cartilage, type II
5   collagen and proteoglycan synthesis were increased after TGF-
β administration. Morales and Roberts, 1988, *J. Biol. Chem.*
263: 12828- 12831. In contrast, the TGF-βs have been shown to
decrease expression of type II and type X cartilage-specific
collagens, synthesis of chondrocyte proteoglycans, and
10 activity of alkaline phosphatase in cultured chondroid cells.
Mundy. "The effects of TGF-β on bone," *Clinical Applications
of TGF-β.*, 1991, Wiley Chichester, Ciba Foundation Symposium
157: 137-151. Rabbit growth plate chondrocyte
differentiation is inhibited by TGF-β, while growth plate
15 chondrocyte mitogenesis is increased. Kato, *et al.*, 1988,
Proc. Natl. Acad. Sci. USA 85: 9552-9556. In addition, large
concentrations of TGF-β1 or TGF-β2 added to an osteoinductive
model favor cartilage, rather than the preference for bone
formation, when smaller doses are used. Mundy, *supra.* This
20 accumulation of apparently contradictory data has hindered
efforts to define a function for the TGF-βs in
chondrogenesis.

          The Bone Morphogenic Proteins And Bone Formation. A
family of proteins termed the bone morphogenetic proteins
25 (BMP's are capable of inducing ectopic bone formation in
certain mammalian species. With the exception of BMP-1,
which encodes a metalloprotease, all of these proteins have
structures that are related to TGF-β. However, it is not
known which, if any, of the BMP's are responsible for the
30 regulation of bone formation during normal embryogenesis.
          BMP's were first isolated from demineralized bone as
factors that induced bone at extra skeletal ectopic sites.
Three peptides were originally identified as BMP-1, BMP-2A,
and BMP-3. Celeste, *et al.*, 1990, *Proc. Natl Acad. Sci. USA*
35 87: 9843-9847; Kubler and Urist, 1990, *Clin. Orthopedics and
Rel. Res.* 258: 279-294. The latter two BMPs are members of
the TGF-β superfamily. Subsequently, five more closely
related members of the BMP group have been identified and

cloned. BMP-5, BMP-6, and BMP-7 are most similar to vgr/60A,
while BMP-2 and BMP-4 are more similar to Decapentaplegic.
Both vga/60A and Decapentaplegic are Drosophila genes that
control dorsal/ventral axis pattern formation. Hoffman,
5   1992, *Mol. Repro and Dev.* 32: 173-178.

In-situ hybridization has localized BMP's gene
transcription to areas of bone formation in the limb bud at
specific times during development, suggesting a physiologic
role. The BMPs induce adventitial post-fetal mesenchymal
10 cells to switch from fibrogenetic to chondroosteoprogenetic
patterning. Kubler and Urist, *supra.* Several lines of data.
suggest the BMPs may act synergistically with TGF-βs to
initiate the cascade of osteoinduction in-vivo. In murine
subcutis, TGF-β1 enhances the production of ectopic bone by
15 most BMPs. BMP-6 (also known as VGR-1) is expressed in
hypertrophic cartilage at the same time and in the same areas
as the TGF-βs, and is associated with collagen type X
expression. *See,* Celeste, *et al., supra.*

The addition of TGFβ-2 to bone explants which have been
20 treated with either BMP-2 or BMP-3 results in increased
osteoinductive activity and an increased ratio of cartilage
to bone when compared to either factor alone. Bentz, *et al.,*
1991, *Matrix* 11:269-275. However, the synergistic effect of
these proteins by the TGF-βs is not universal. TGF-β1 has
25 been shown to directly decrease BMP-2 expression in fetal rat
calvaria cultures. Harris, *et al.,* 1994, *J. Bone and Mineral
Res.* 9: 855-863. Since BMP-2 is apparently important in bone
cell differentiation, it has been suggested that TGF-β1 may
be acting as a switch to monitor the differentiation fates of
30 chondro- or osteo- blastic precursors.

*Other Factors Found To Be Expressed In Developing
Tissue.* Cyr61 is a growth regulator which has been found to
be expressed in developing mouse embryo and extraembryonic
tissues. O'Brien and Lau, 1992, *Cell Growth Differ.* 3:645-
35 654. Cyr61 is related to but distinct from CTGF and prior to
the instant invention, the specific activity of Cyr61 was not
known.

- 5 -

B.   The Role Of Growth Factors In Wound Healing

      Platelet Derived Growth Factor And Wound Healing.
PDGF is a dimeric molecule consisting of an A chain and a B
chain. The chains form heterodimers of homodimers and all
combinations isolated to date are biologically active. With
respect to the factor's activity, PDGF has been characterized
as a cationic, heat-stable protein found in the α-granules of
circulating platelets. The molecule has been further
characterized as a mitogen and a chemotactic agent for
connective tissue cells such as fibroblasts and smooth muscle
cells.

      Because of PDGF's biological activity and release during
wound healing, PDGF has been identified as a growth factor
involved in wound healing, as well as pathological conditions
showing on overproduction of connective tissue, including
atherosclerosis and fibrotic diseases.

      It has been hypothesized that growth factors other than
PDGF may play a role in the normal development, growth, and
repair of human tissue.

      TGF-β And Wound Healing. The formation of new and
regenerating tissue requires the coordinate regulation of
various genes that produce both regulatory and structural
molecules which participate in the process of cell growth and
tissue organization. As with bone induction, it appears that
TGF-β plays a central regulatory component of this process.
TGF-β is released by platelets, macrophages and neutrophils
which are present in the initial phases of the repair
process. TGF-β can act as a growth stimulatory factor for
mesenchymal cells and as a growth inhibitory factor for
endothelial and epithelial cells. It has been suggested that
the growth stimulatory action of TGF-β appears to be mediated
via an indirect mechanism involving the induction of other
genes including growth factors such as PDGF.

      Several members of the TGF-β superfamily possess
activities suggesting possible applications for the treatment
of cell proliferative disorders, such as cancer. In
particular, TGF-β has been shown to be potent growth
inhibitor for a variety of cell types (Massague, 1987, *Cell*

- 6 -

49:437), MIS has been shown to inhibit the growth of human
endometrial carcinoma tumors in nude mice (Donahoe, et al.,
1981, Ann. Surg. 194:472), and inhibition has been shown to
suppress the development of tumors both in the ovary and in
5   the testis (Matzuk, et al., 1992, Nature, 360:313).

        Many of the members of the TGF-β family are also
important mediators of tissue repair. TGF-β has been shown
to have marked effects on the formation of collagen and
causes of striking angiogenic response in the newborn mouse
10   (Roberts, et al., 1986, Proc. Natl. Acad. Sci., USA 83:4167).
The bone morphogenic proteins (BMPs) can induce new bone
growth and are effective for the treatment of fractures and
other skeletal defects (Glowacki, et al., 1981 Lancet, 1:959;
Ferguson, et al., 1988, Clin. Orthoped. Relat. Res., 227:265;
15   Johnson, et al., 1988, Clin. Orthoped. Relat. Res., 230:257).

        C.   Connective Tissue Growth Factor
              A previously unknown growth factor, related to
PDGF, and termed Connective Tissue Growth Factor (CTGF), has
20   been reported in a related patent. See, U.S. Patent No.
5,408,040. CTGF is a cysteine-rich mitogenic peptide which
is selectively induced in fibroblasts after activation with
TGF-β. Igarashi, et al., 1993, Mol. Biol. Cell 4:637-645.

        CTGF is a member of a family of peptides that include
25   serum induced gene products cef10 (Simmons, et al., 1989,
Proc. Natl. Acad. Sci. USA 86:1178-1182), cyr61 (O'Brien, et
al., 1990, Mol. Cell. Biol. 10:3569-3577), fisp12/IG M1
(Ryseck, et al., 1993, Cell Growth & Differ. 2:225-233), and
a chicken transforming gene, nov (Joliot, et al. 1992, Mol.
30   Cell Biol. 12:10-21 (1992). CTGF also shares sequence
homology with a drosophila gene product, twisted gastrulation
(twg) (Mason, et al.., 1994, Genes & Develop. 8:1489-1501),
which determines cell fates during dorsal/ventral pattern
formation in the embryo.

35   As reported in that patent, CTGF is the product of a
distinct gene. As also reported in U.S. Patent No.
5,408,040, CTGF possesses mitogenic activity. The ultimate
result of this mitogenic activity in vivo, is the growth of

                            - 7 -

targeted tissue. CTGF also possesses chemotactic activity,
which is the chemically induced movement of cells as a result
of interaction with particular molecules.

    Although the molecule is antigenically related to PDGF,
5   there is little if any peptide sequence homology between CTGF
and PDGF. Anti-PDGF antibody has high affinity to the non-
reduced forms of the PDGF isomers and the CTGF molecule and
ten-fold less affinity to the reduced forms of these
peptides, which lack biological activity.

10   A second protein, identified as "connective tissue
growth factor-2" or "CTGF-2," has been reported also. See,
PCT Application No. PCT/US94/07736 (International Publication
No. WO 96/01896). According to the PCT Application, CTGF-2
may also be used to enhance the repair of connective and
15 support tissue. Although identified as a connective tissue
growth factor, CTGF-2 is not closely related to the CTGF of
the present invention. Specifically, the CTGF family is
comprised of three distinct groups of proteins: CTGF/Fisp12,
cyr61 and nov. The protein of the claimed invention falls
20 within the first group of proteins, as compared to CTGF-2,
which falls with the cyr61 group. PCT Application No.
PCT/US94/07736 at 4.

    Notwithstanding the identification of various PDGF
related growth factors, including CTGF, prior to the present
25 invention, such factors have not been proven to be an
effective induction agent for the production of matrices,
including the induction of bone and/or cartilage tissue.

4.   **SUMMARY OF THE INVENTION**
30   The subject invention provides novel methods and
compositions for the treatment of diseases, disorders or
ailments wherein matrix and/or connective tissue production,
including the production of bone and/or cartilage, is
desired. The subject invention is likewise directed to the
35 treatment of diseases, disorders or ailments wherein the
promotion of wound healing is desired.

    More specifically, the compositions of the present
invention comprise CTGF and/or fragments and/or derivatives

- 8 -

thereof (hereinafter collectively "CTGF"), alone or in
combination with other growth factors. The CTGF used in the
subject compositions may be either obtained by isolation from
natural sources, synthetic manufacture, or production by
5   recombinant genetic engineering techniques.
        In one aspect of the invention, the methods of the
present invention comprise the administration of an effective
amount of CTGF, alone or in combination with one or more
compounds, to treat diseases, disorders or ailments wherein
10 the induction of bone or cartilage tissue is desired. In a
preferred embodiment of this method, such additional compound
is a growth factor.
        In another aspect of the invention, the methods of the
present invention comprise the administration of an effective
15 amount of CTGF, alone or in combination with one or more
compounds, again preferably one or more growth factors, to
treat diseases, disorders or ailments wherein the promotion
of wound healing is desired.
        In a preferred embodiment of the invention, the
20 composition comprising CTGF is administered directly onto or
into the site in which bone or cartilage induction is desired
so as to induce the formation of such bone or cartilage. In
another embodiment, the composition is formulated for
targeted delivery or alternatively, are designed for the
25 release of the novel compositions in the relevant site (e.g.,
the wound in which cartilage formation is desired). In each
case, the CTGF containing composition is appropriately
formulated for administration to a patient in need.

30 5.   DEFINITIONS
        As used in this Specification, the term "CTGF" shall
mean: (1) a protein encoded by the amino acid sequence set
forth at Figure 1C, (2) a protein having CTGF activity
wherein such protein is encoded by the amino acid sequence of
35 Figure 1C wherein one or more amino acids have been added,
deleted, mutated, substituted or otherwise altered
("derivative") and the nucleotide sequence encoding said
protein can hybridize to the nucleic acid sequence of Figure

- 9 -

1C under stringent conditions, or (3) a fragment of CTGF or a
derivative thereof.

   As used in this Specification, the term "induce," as
used herein, shall mean to produce, form, to cause to
5 produce, or to cause to form.

   As used in this Specification, the phrase "induction
agent" shall mean an agent, including proteins or other
biological materials, which causes the production or
formation of a specific end result (e.g., the production of
10 connective tissue).

   As used in this Specification, the term "polynucleotide"
denotes DNA, cDNA and/or RNA which encode untranslated
sequences which flank the structural gene encoding CTGF. For
example, a polynucleotide of the invention includes 5'
15 regulatory nucleotide sequences and 3' untranslated sequences
associated with the CTGF structural gene. A polynucleotide
of the invention which includes the 5' and 3' untranslated
region is illustrated in FIGURE 1C. The 5' regulatory
region, including the promoter, is illustrated in FIGURE 1B.
20 A more detailed description of the polynucleotides
contemplated by the present invention may be found at U.S.
Patent No. 5,408,040.

   As used in this Specification, the phrase "stringent
conditions," as used herein, refers to those hybridizing
25 conditions that (1) employ low ionic strength and high
temperature for washing, for example, 0.015 M NaCl/0.0015 M
sodium citrate/0.1% SDS at 50°C.; (2) employ during
hybridization a denaturing agent such as formamide, for
example, 50% (vol/vol) formamide with 0.1% bovine serum
30 albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium
phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium
citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M
NaCl, 0.075 M Sodium pyrophosphate, 5 x Denhardt's solution,
sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10%
35 dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and
0.1% SDS.

   As used in this Specification, the phrase "recombinant
expression vector" refers to a plasmid, virus or other

                          - 10 -

vehicle known in the art that has been manipulated by
insertion or incorporation of the CTGF genetic sequences.

As used in this Specification, the phrase
"therapeutically effective" means that amount of CTGF which
5   is effective in inducing bone or cartilage formation or wound
healing.

6.   BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A shows the structural organization of the CTGF
10   gene. Exons are indicated by boxed regions, with solid areas
in the gene corresponding to the open reading frame.

FIGURE 1B shows a comparison of nucleotide sequences
between CTGF promoter and fisp-12 promoter. Identical
nucleotides are marked with asterisks. The TATA box and
15   other consensus sequences are indicated and shaded. The site
of transcriptional initiation is indicated at position number
+1.

FIGURE 1C shows the complete nucleotide and deduced
amino acid sequence for the CTGF structural gene and 5' and
20   3' untranslated sequences.

FIGURE 2 shows experimental *in situ* hybridization
results related to the expression of CTGF transcripts in the
growth plate of long bones in newborn mice. The *in situ*
hybridization experiments were performed using an anti-sense
25   CTGF RNA probe as described below. Chondrocytes in the
proliferation zone are strongly positive for CTGF gene
expression, indicating that CTGF is produced at sites of
cartilage growth.

FIGURE 3 shows the expression of the CTGF gene during
30   embryogenesis wherein a transgenic mouse is constructed using
a fusion gene constructed from a CTGF promoter and a β-
galactosidase structural gene. This gene, introduced into
the germ line, expresses β-galactosidase at sites of CTGF
expression and can be detected by histochemical means by
35   expressing sections of the developing transgenic animal to
the substrate X-gal which deposits a blue color at sites of
β-galactosidase activity. Panel A is a 12 day mouse embryo
from such a transgenic mouse. The blue staining is an area

- 11 -

destined to become Meckel's cartilage, which is the first
cartilage to form. Panel B is a photograph of the hind limb
and paw which demonstrates staining at the ends of the long
bones and in the paw in the growth plates of the metatarsal.

5   FIGURE 4 provides evidence of induction of cartilage and
bone in cultures of C3H10T1/2 mouse embryonic stem cells.
C3H10T1/2 cells were cultured as described under methods.
Cells were treated with either nothing (Panel A), 5 -
azacytodine (Panel B), CTGF at 50 ng/ml (Panel C) or 5-

10   azacytodine followed by CTGF (Panel D).
        FIGURE 5 sets forth Northern blot analysis of CTGF gene
expression in wound chambers implanted at sites of bone
regeneration.
        FIGURE 6 sets forth evidence related to the expression

15   of CTGF in human osteoblasts in response to TGF-β.
        FIGURES 7A-7D set forth results of a chondrogenic assay.
            FIGURE 7A provides the chondrogenic assay results
for the control culture.
            FIGURE 7B provides the chondrogenic assay results

20   for a culture in which 5 ng/ml TGF-β1 was added.
            FIGURE 7C provides the chondrogenic assay results
for a culture in which 5 ng/ml TGF-β1 and 10 ng cholera toxin
were added.
            FIGURE 7D provides the chondrogenic assay results

25   for a culture in which 5 ng/ml TGF-β1, 10 ng/ml cholera
toxin, and 5 ng/ml CTGF were added.
        FIGURE 8A is a Scatchard Plot reflecting CTGF binding to
NRK cells.
        FIGURE 8B is a Sctachard Plot reflecting CTGF binding to

30   rat chondroblasts.

    7.   DETAILED DESCRIPTION OF THE INVENTION
        7.1. Methods For Making CTGF
            Nucleic Acid Sequences Encoding CTGF.. In

35   accordance with the invention, nucleotide sequences encoding
CTGF or functional equivalents thereof may be used to
generate recombinant DNA molecules that direct the expression
of the protein or a functional equivalent thereof, in

appropriate host cells. Alternatively, nucleotide sequences
which hybridize, under stringent position, to portions of the
CTGF sequence may also be used in nucleic acid hybridization
assays, Southern and Northern blot analyses, etc. In yet
5 another method, DNA molecules encoding CTGF may be isolated
by hybridization procedures comprising antibody screening of
expression libraries to detect shared structural features.

Due to the inherent degeneracy of the genetic code,
other DNA sequences which encode substantially the same or a
10 functionally equivalent amino acid sequence, may be isolated
and used in the practice of the invention for the cloning and
expression of CTGF. Such DNA sequences include those which
are capable of hybridizing to the human CTGF sequence under
stringent conditions.

15 Altered DNA sequences which may be used in accordance
with the invention include deletions, additions or
substitutions of different nucleotide residues resulting in a
sequence that encodes the same or a functionally equivalent
gene product. The gene product itself may contain deletions,
20 additions or substitutions of amino acid residues within the
CTGF sequence, which result in a silent change thus producing
a functionally equivalent protein. Such amino acid
substitutions may be made on the basis of similarity in
polarity, charge, solubility, hydrophobicity, hydrophilicity,
25 and/or the amphipatic nature of the residues involved. For
example, negatively charged amino acids include aspartic acid
and glutamic acid; positively charged amino acids include
lysine and arginine; amino acids with uncharged polar head
groups having similar hydrophilicity values include the
30 following: leucine, isoleucine, valine; glycine, analine;
asparagine, glutamine; serine, threonine; phenylalanine,
tyrosine.

The DNA sequences of the invention may be engineered in
order to alter the protein's sequence for a variety of ends
35 including but not limited to alterations which modify
processing and expression of the gene product. For example,
mutations may be introduced using techniques which are well
known in the art, e.g., site-directed mutagenesis to, for

- 13 -

example, insert new restriction sites. For example, in
certain expression systems such as yeast, host cells may
over- glycosylate the gene product. When using such
expression systems it may be preferable to alter CTGF coding
5   sequence to eliminate any N-linked glycosylation site.

The CTGF sequence may be ligated to a heterologous
sequence to encode a fusion protein. For example, for
screening of peptide libraries it may be useful to encode a
chimeric CTGF protein expressing a heterologous epitope that
10   is recognized by a commercially available antibody. A fusion
protein may also be engineered to contain a cleavage site
located between the CTGF sequence and the heterologous
protein sequence (e.g. a sequence encoding a growth factor
related to PDGF), so that CTGF can be cleaved away from the
15   heterologous moiety.

The coding sequence of CTGF may also be synthesized in
whole or in part, using chemical methods well known in the
art. See, for example, Caruthers, et al., 1980, *Nucleic
Acids Res. Symp. Ser.* 7:215-233; Crea and Horn, 1980, *Nucleic*
20   *Acids Res.* 9(10):2331; Matteucci and Caruthers, 1980,
Tetrahedron Letters 21:719; and Chow and Kempe, 1981, *Nucleic*
Acids Res. 9(12):2807-2817. Alternatively, the protein
itself could be produced using chemical methods to synthesize
the CTGF amino acid sequence in whole or in part. For
25   example, peptides can be synthesized by solid phase
techniques, cleaved from the resin, and purified by
preparative high performance liquid chromatography. *See*
e.g., Creighton, 1983, *Proteins Structures And Molecular*
Principles, W.H. Freeman and Co., N.Y. pp. 50-60. The
30   composition of the synthetic peptides may be confirmed by
amino acid analysis or sequencing. See e.g., for the Edman
degradation procedure, *see*, Creighton, 1983, *Proteins,*
Structures and Molecular Principles, W.H. Freeman and Co.,
N.Y., pp. 34-49.

35   A more detailed description of the nucleic acid
sequences comprising the present invention and methods for
identifying such sequences may be found in U.S. Patent,

- 14 -

Serial No. 5,408,040, which is incorporated herein by
reference.

           Expression Of CTGF. In order to express a
biologically active CTGF, the nucleotide sequence coding for
5   the protein, or a functional equivalent as described above,
supra, was inserted into an appropriate expression vector,
i.e., a vector which contains the necessary elements for the
transcription and translation of the inserted coding
sequence.

10   More specifically, methods which are well known to those
skilled in the art can be used to construct expression
vectors containing the CTGF sequence and appropriate
transcriptional/translational control signals. These methods
include in vitro recombinant DNA techniques, synthetic
15   techniques and in vivo recombination/genetic recombination.
See e.g., the techniques described in Maniatis *et al.*, 1989,
Molecular Cloning: A Laboratory Manual, Cold Spring Harbor
Laboratory, N.Y. and Ausubel *et al.*, 1989, Current Protocols
in Molecular Biology, Greene Publishing Associates and Wiley
20   Interscience, N.Y.

          A variety of host-expression vector systems may be
utilized to express the CTGF coding sequence. These include
but are not limited to microorganisms such as bacteria
transformed with recombinant bacteriophage DNA, plasmid DNA
25   or cosmid DNA expression vectors containing the CTGF coding
sequence; yeast, including *Pichia pastoris* and *Hansenula
polymorpha*, transformed with recombinant expression vectors
containing the CTGF coding sequence; insect cell systems
infected with recombinant virus expression vectors (e.g.,
30   bacculovirus) containing the CTGF coding sequence; plant cell
systems infected with recombinant virus expression vectors
(e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus,
TMV) or transformed with recombinant plasmid expression
vectors (e.g., Ti plasmid) containing the CTGF coding
35   sequence; or animal cell systems infected with recombinant
virus expression vectors (e.g., adenovirus, vaccinia virus,
human tumor cells (including HT-1080)) including cell lines
engineered to contain multiple copies of the CTGF DNA either

                                - 15 -

stably amplified (CHO/dhfr) or unstably amplified in double-
minute chromosomes (e.g., murine cell lines). As used
herein, it is understood that the term "host-expression
vector systems" and more generally, the term "host cells"
includes any progeny of the host cell or host-expression
vector system. It is further understood that although all
progeny may not be identical to the parental cell, as
mutations may occur during replication, such progeny are
included in the scope of the invention.

The expression elements of these systems vary in their
strength and specificities. Depending on the host/vector
system utilized, any of a number of suitable transcription
and translation elements, including constitutive and
inducible promoters, may be used in the expression vector.
For example, when cloning in bacterial systems, inducible
promoters such as pL of bacteriophage λ, plac, ptrp, ptac
(ptrp-lac hybrid promoter) and the like may be used; when
cloning in insect cell systems, promoters such as the
bacculovirus polyhedrin promoter may be used; when cloning in
plant cell systems, promoters derived from the genome of
plant cells (e.g., heat shock promoters; the promoter for the
small subunit of RUBISCO; the promoter for the chlorophyll
a/b binding protein) or from plant viruses (e.g., the 35S RNA
promoter of CaMV; the coat protein promoter of TMV) may be
used; when cloning in mammalian cell systems, promoters
derived from the genome of mammalian cells (e.g.,
metallothionein promoter) or from mammalian viruses (e.g.,
the adenovirus late promoter; the vaccinia virus 7.5K
promoter) may be used; when generating cell lines that
contain multiple copies of the CTGF DNA SV40-, BPV- and EBV-
based vectors may be used with an appropriate selectable
marker.

In bacterial systems, a number of expression vectors may
be advantageously selected depending upon the use intended
for the expressed CTGF. For example, a suitable vector for
expression in bacteria includes the T7-based vector as
described in Rosenberg, et al.., 1987, *Gene* 56:125. As
further example, when large quantities of CTGF are to be

- 16 -

produced to screen peptide libraries, vectors which direct
the expression of high levels of protein products that are
readily purified may be desirable. Such vectors include but
are not limited to the E. coli expression vector pUR278
(Ruther et al., 1983, EMBO J. 2:1791), in which the CTGF
coding sequence may be ligated into the vector in frame with
the lac Z coding region so that a hybrid AS-lac Z protein is
produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids
Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem.
264:5503-5509); and the like. pGEX vectors may also be used
to express foreign polypeptides such as CTGF with glutathione
S-transferase (GST). In general, such fusion proteins are
soluble and can easily be purified from lysed cells by
adsorption to glutathione-agarose beads followed by elution
in the presence of free glutathione. The pGEX vectors are
designed to include thrombin or factor Xa protease cleavage
sites so that the cloned polypeptide of interest can be
released from the GST moiety.

    More generally, where the host is a procarote, competent
cells which are capable of DNA uptake can be prepared from
cells harvested after exponential growth and subsequently
treated by the CaCl$_2$, or alternatively MgCl$_2$ or RbCl, method
using procedures well known in the art.

    Where the host cell is a eucaryote, various methods of
DNA transfer can be used. These include transfection of DNA
by calcium phosphate-precipitates, conventional mechanical
procedures, including microinjection, insertion of a plasmid
encased in liposomes, or use of virus vectors. Eucaryotic
cells may also be cotransformed with DNA sequences encoding
the polypeptide of the invention, and a second foreign DNA
molecule encoding a selectable phenotype, such as herpes
simplex thymidine kinase gene. Another method is to use a
eukaryotic viral vector, such as simian virus 40 (SV40) or
bovine papilloma virus, to transiently infect or transform
eucaryotic cells and express protein. See, Eukaryotic Viral
Vectors, 1992, Cold Spring Harbor Laboratory, Gluzman, Ed.).
Eucaryotic host cells include yeast, mammalian cells, insect
cells and plant cells.

- 17 -

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ausubel et al., Ed., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Methods in Enzymology, Wu & Grossman, Eds., Acad. Press, N.Y., 153:516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Berger & Kimmel, Eds., Acad. Press, N.Y., 152:673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Strathern et al., Eds., Cold Spring Harbor Press, Vols. I and II. For example, various shuttle vectors for the expression of foreign genes in yeast have been reported. Heinemann, et al., 1989, Nature 340:205; Rose, et al., 1987, Gene 60:237.

In cases where plant expression vectors are used, the expression of the CTGF coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques, see, e.g., Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

In an insect system, an alternative expression system could be used to express CTGF. In one such system, Bacculovirus is used as a vector to express foreign genes. The virus then grows in the insect cells. The CTGF coding sequence may be cloned into non-essential regions (for

- 18 -

example the polyhedrin gene) of the virus and placed under
control of a Bacculovirus promoter. These recombinant
viruses are then used to infect insect cells in which the
inserted gene is expressed. See, e.g., Smith et al., 1983,
5   J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051.
          In mammalian host cells, a number of viral based
expression systems may be utilized. In cases where an
adenovirus is used as an expression vector, the CTGF coding
sequence may be ligated to an adenovirus
10   transcription/translation control complex, e.g., the late
promoter and tripartite leader sequence. This chimeric gene
may then be inserted in the adenovirus genome by in vitro or
in vivo recombination. Insertion in a non-essential region
of the viral genome (e.g., region E1 or E3) will result in a
15   recombinant virus that is viable and capable of expressing
CTGF in infected hosts. See e.g., Logan & Shenk, 1984, Proc.
Natl. Acad. Sci. (USA) 81:3655-3659. Alternatively, the
vaccinia 7.5K promoter may be used. See, e.g., Mackett et
al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett
20   et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982,
Proc. Natl. Acad. Sci. 79:4927-4931.
          In another embodiment, the CTGF sequence is expressed in
human tumor cells, such as HT-1080, which have been stably
transfected with calcium phosphate precipitation and a
25   neomycin resistance gene. In yet another embodiment, the
pMSXND expression vector or the like is used for expression
in a variety of mammalian cells, including COS, BHK 293 and
CHO cells. Lee and Nathans, 1988, J. Biol. Chem. 263:3521.
          Specific initiation signals may also be required for
30   efficient translation of inserted CTGF coding sequences.
These signals include the ATG initiation codon and adjacent
sequences. In cases where the entire CTGF gene, including
its own initiation codon and adjacent sequences, is inserted
into the appropriate expression vector, no additional
35   translational control signals may be needed. However, in
cases where only a portion of the CTGF coding sequence is
inserted, exogenous translational control signals, including
the ATG initiation codon, must be provided. Furthermore, the

- 19 -

initiation codon must be in phase with the reading frame of
the CTGF coding sequence to ensure translation of the entire
insert. These exogenous translational control signals and
initiation codons can be of a variety of origins, both
5   natural and synthetic. The efficiency of expression may be
enhanced by the inclusion of appropriate transcription
enhancer elements, transcription terminators, etc. See e.g.,
Bitter et al., 1987, Methods in Enzymol. 153:516-544.

In addition, a host cell strain may be chosen which
10 modulates the expression of the inserted sequences, or
modifies and processes the gene product in the specific
fashion desired. Such modifications (e.g., glycosylation)
and processing (e.g., cleavage) of protein products may be
important for the function of the protein. Different host
15 cells have characteristic and specific mechanisms for the
post-translational processing and modification of proteins.
Appropriate cells lines or host systems can be chosen to
ensure the correct modification and processing of the foreign
protein expressed. To this end, eukaryotic host cells which
20 possess the cellular machinery for proper processing of the
primary transcript, glycosylation, and phosphorylation of the
gene product may be used. Such mammalian host cells include
but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293,
WI38, HT-1080 etc.

25   For long-term, high-yield production of recombinant
proteins, stable expression is preferred. For example, cell
lines which stably express CTGF may be engineered. Rather
than using expression vectors which contain viral origins of
replication, host cells can be transformed with CTGF DNA
30 controlled by appropriate expression control elements (e.g.,
promoter, enhancer, sequences, transcription terminators,
polyadenylation sites, etc.), and a selectable marker.
Following the introduction of foreign DNA, engineered cells
may be allowed to grow for 1-2 days in an enriched media, and
35 then are switched to a selective media. The selectable
marker in the recombinant plasmid confers resistance to the
selection and allows cells to stably integrate the plasmid

- 20 -

into their chromosomes and grow to form foci which in turn
can be cloned and expanded into cell lines.

A number of selection systems may be used, including but
not limited to the herpes simplex virus thymidine kinase
5   (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine
phosphoribosyltransferase (Szybrlska & Szybalski, 1962, Proc.
Natl. Acad. Sci. (USA) 48:2026), and adenine
phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817)
genes can be employed in tk⁻, hgprt⁻ or aprt⁻ cells,
10   respectively.

Also, antimetabolite resistance can be used as the basis
of selection for dhfr, which confers resistance to
methotrexate (Wigler, et al., 1980, Proc. Natl. Acad. Sci.
(USA) 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci.
15   (USA) 78:1527); gpt, which confers resistance to mycophenolic
acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. (USA)
78:2072); neo, which confers resistance to the aminoglycoside
G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1);
and hygro, which confers resistance to hygromycin (Santerre,
20   et al., 1984, Gene 30:147) genes. Recently, additional
selectable genes have been described, namely trpB, which
allows cells to utilize indole in place of tryptophan; hisD,
which allows cells to utilize histinol in place of histidine
(Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. (USA)
25   85:8047), and ODC (ornithine decarboxylase) which confers
resistance to the ornithine decarboxylase inhibitor, 2-
(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In:
Current Communications in Molecular Biology, Cold Spring
Harbor Laboratory).

30   The isolation and purification of host cell expressed
polypeptides of the invention may be by any conventional
means such as, for example, preparative chromatographic
separations and immunological separations such as those
involving the use of monoclonal or polyclonal antibody.

35

- 21 -

7.2. Identification Of Transfectants Or Transformants That Express CTGF

The host cells which contain the coding sequence
5   and which express the biologically active gene product may be
identified by at least four general approaches: (a) DNA-DNA
or DNA-RNA hybridization; (b) the presence or absence of
"marker" gene functions; (c) assessing the level of
transcription as measured by the expression of CTGF mRNA
10 transcripts in the host cell; and (d) detection of the gene
product as measured by an assay or by its biological
activity.

In the first approach, the presence of the CTGF coding
sequence inserted in the expression vector can be detected by
15 DNA-DNA or DNA-RNA hybridization using probes comprising
nucleotide sequences that are homologous to the CTGF coding
sequence, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression
vector/host system can be identified and selected based upon
20 the presence or absence of certain "marker" gene functions
(e.g., resistance to antibiotics, resistance to methotrexate,
transformation phenotype, occlusion body formation in
bacculovirus, etc.). For example, in a preferred embodiment,
the CTGF coding sequence is inserted within a neomycin-
25 resistance marker gene sequence of the vector, and
recombinants containing the CTGF coding sequence can be
identified by the absence of the marker gene function.
Alternatively, a marker gene can be placed in tandem with the
CTGF sequence under the control of the same or different
30 promoter used to control the expression of the CTGF coding
sequence. Expression of the marker in response to induction
or selection indicates expression of the CTGF coding
sequence.

In the third approach, transcriptional activity for the
35 CTGF coding region can be assessed by hybridization assays.
For example, RNA can be isolated and analyzed by Northern
blot using a probe homologous to the CTGF coding sequence or
particular portions thereof. Alternatively, total nucleic

acids of the host cell may be extracted and assayed for
hybridization to such probes.

     The fourth approach involves the detection of the
biologically active or immunologically reactive CTGF gene
5   product. A number of assays can be used to detect CTGF
activity including but not limited to those assays described
in U.S. Patent No. 5,408,040.

     ### 7.3. Treatment Indications
10   The methods, compounds and formulations of the
present invention are each directed to the treatment of
disorders, diseases or ailments related to the
underproduction of connective tissue in bone, cartilage, or
other organs such as skin and muscle alternatively, to
15   disorders, diseases or ailments in which the formation of
bone or cartilage is desired.

     These diseases, disorders or ailments include the repair
of cartilage or bone defect after a variety of traumatic
injuries or disorders including arthritis, osteoporosis and
20   other skeletal disorders, hypertrophic scars, burns, and
vascular hypertrophy. Because these problems are due to a
poor growth response of the fibroblasts, stem cells,
chondrocytes, osteoblasts or fibroblasts at the site of
injury, the addition of a biologically active agent which
25   could stimulate the growth of these cells would be
beneficial.

     Another important use of CTGF would be in culture
systems to expand stem cells or chondrocytes that were
removed from an individual prior to reimplantation. In a
30   similar process, CTGF could be added to either stem cells or
chondrocytes when they were to be added as a graft to help
stimulate the expansion and differentiation of these cells at
the site of implantation. CTGF could also be added to a
graft composed of cartilage or bone to help stimulate growth.
35   Another treatment indication is directed to
administering CTGF to a patient in need to enhance wound
healing. PDGF and other growth factors, such as CTGF, are
involved in normal healing of skin wounds. The CTGF

polypeptide of this invention is valuable as a therapeutic in
cases in which there is impaired healing of skin wounds or
there is a need to augment the normal healing mechanisms,
e.g., burns. One important advantage to using CTGF protein
5   to accelerate wound healing is attributable to the molecule's
high percentage of cysteine residues. CTGF, or functional
fragments thereof, is more stable and less susceptible to
protease degradation than PDGF and other growth factors known
to be involved in wound healing.
10   Preferably, the agent of this invention is the
combination of TGF-β and CTGF, however, it is likely that
other TGF-β family members will also be useful in
accelerating wound healing by inducing CTGF. The composition
of the invention aids in healing the wound, in part, by
15   promoting the growth of connective tissue. The composition
is prepared by combining, in a pharmaceutically acceptable
carrier substance, e.g., inert gels or liquids, the purified
CTGF and TGF-β.
         The treatment indications, with respect to wound
20   healing, contemplated by this invention include anticipated
wounds (i.e. wounds resulting from surgical procedures), as
well as unanticipated wounds (i.e. wounds caused by trauma).

         7.4. Pharmaceutical Formulations And Routes Of
25   **Administration**

             The molecules of the present invention can be
administered to a patient in need, by itself, or in
pharmaceutical compositions where one or more of the
30   molecules are mixed with suitable carriers or excipient(s) at
doses to treat or ameliorate a variety of disorders.
Alternatively, as CTGF is produced by endothelial cells and
fibroblastic cells, both of which are present at the site of
bone or cartilage formation and wounding, agents which
35   stimulate the production of CTGF can be added to a
composition which is used to accelerate bone or cartilage
induction or wound healing. Preferably, the agent of this
invention is transforming growth factor beta. The
composition of the invention aids in healing the wound, in

- 24 -

part, by promoting the growth of connective tissue. In
another embodiment, CTGF may be administered in combination
with proteins or compounds which are believed to promote the
formation of connective tissue.

5   Whether the composition is comprised of CTGF alone or
CTGF and additional agents as the active ingredient, such
composition is prepared by combining, in a pharmaceutically
acceptable carrier substance, e.g., inert gels or liquids,
the purified CTGF and TGF-β.

10   A therapeutically effective dose further refers to that
amount of the compound sufficient to result in amelioration
of symptoms. Techniques for formulation and administration
of the compounds of the instant application may be found in
"Remington's Pharmaceutical Sciences," Mack Publishing Co.,
15   Easton, PA, latest edition.

               7.4.1.   Routes Of Administration.
               Suitable routes of administration may, for example,
       include oral, rectal, transmucosal, or intestinal
20   administration; parenteral delivery, including intramuscular,
       subcutaneous, intramedullary injections, as well as
       intrathecal, direct intraventricular, intravenous,
       intraperitoneal, intranasal, or intraocular injections.
               Alternately, one may administer the compound in a local
25   rather than systemic manner, for example, via injection of
       the compound directly into an area requiring CTGF, often in a
       depot or sustained release formulation.
               Furthermore, one may administer the drug in a targeted
       drug delivery system, for example, in a liposome coated with
30   a specific antibody, targeting, for example, cartilage. The
       liposomes will be targeted to and taken up selectively by the
       afflicted tissue.

               7.4.2.   Composition/Formulation.
35   The pharmaceutical compositions of the present
       invention may be manufactured in a manner that is itself
       known, e.g., by means of conventional mixing, dissolving,

granulating, dragee-making, levigating, emulsifying,
encapsulating, entrapping or lyophilizing processes.

       Pharmaceutical compositions for use in accordance with
the present invention thus may be formulated in conventional
manner using one or more physiologically acceptable carriers
comprising excipients and auxiliaries which facilitate
processing of the active molecules into preparations which
can be used pharmaceutically. Proper formulation is
dependent upon the route of administration chosen.

       For injection, the agents of the invention may be
formulated in aqueous solutions, preferably in
physiologically compatible buffers such as Hanks's solution,
Ringer's solution, or physiological saline buffer. For
transmucosal administration, penetrants appropriate to the
barrier to be permeated are used in the formulation. Such
penetrants are generally known in the art.

       For oral administration, the compounds can be formulated
readily by combining the active compounds with
pharmaceutically acceptable carriers well known in the art.
Such carriers enable the compounds of the invention to be
formulated as tablets, pills, dragees, capsules, liquids,
gels, syrups, slurries, suspensions and the like, for oral
ingestion by a patient to be treated. Pharmaceutical
preparations for oral use can be obtained solid excipient,
optionally grinding a resulting mixture, and processing the
mixture of granules, after adding suitable auxiliaries, if
desired, to obtain tablets or dragee cores. Suitable
excipients are, in particular, fillers such as sugars,
including lactose, sucrose, mannitol, or sorbitol; cellulose
preparations such as, for example, maize starch, wheat
starch, rice starch, potato starch, gelatin, gum tragacanth,
methyl cellulose, hydroxypropylmethyl-cellulose, sodium
carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP).
If desired, disintegrating agents may be added, such as the
cross-linked polyvinyl pyrrolidone, agar, or alginic acid or
a salt thereof such as sodium alginate.

       Dragee cores are provided with suitable coatings. For
this purpose, concentrated sugar solutions may be used, which

- 26 -

may optionally contain gum arabic, talc, polyvinyl
pyrrolidone, carbopol gel, polyethylene glycol, and/or
titanium dioxide, lacquer solutions, and suitable organic
solvents or solvent mixtures. Dyestuffs or pigments may be
added to the tablets or dragee coatings for identification or
to characterize different combinations of active compound
doses.

     Pharmaceutical preparations which can be used orally
include push-fit capsules made of gelatin, as well as soft,
sealed capsules made of gelatin and a plasticizer, such as
glycerol or sorbitol. The push-fit capsules can contain the
active ingredients in admixture with filler such as lactose,
binders such as starches, and/or lubricants such as talc or
magnesium stearate and, optionally, stabilizers. In soft
capsules, the active compounds may be dissolved or suspended
in suitable liquids, such as fatty oils, liquid paraffin, or
liquid polyethylene glycols. In addition, stabilizers may be
added. All formulations for oral administration should be in
dosages suitable for such administration.

     For buccal administration,the compositions may take the
form of tablets or lozenges formulated in conventional
manner.

     For administration by inhalation, the compounds for use
according to the present invention are conveniently delivered
in the form of an aerosol spray presentation from pressurized
packs or a nebuliser, with the use of a suitable propellant,
e.g., dichlorodifluoromethane, trichlorofluoromethane,
dichlorotetrafluoroethane, carbon dioxide or other suitable
gas. In the case of a pressurized aerosol the dosage unit
may be determined by providing a valve to deliver a metered
amount. Capsules and cartridges of, e.g., gelatin for use in
an inhaler or insufflator may be formulated containing a
powder mix of the compound and a suitable powder base such as
lactose or starch.

     The molecules may be formulated for parenteral
administration by injection, e.g., by bolus injection or
continuous infusion. Formulations for injection may be
presented in unit dosage form, e.g., in ampoules or in multi-

- 27 -

dose containers, with an added preservative. The
composition may take such forms as suspensions, solutions or
emulsions in oily or aqueous vehicles, and may contain
formulatory agents such as suspending, stabilizing and/or
5   dispersing agents.

Pharmaceutical formulations for parenteral
administration include aqueous solutions of the active
compounds in water-soluble form. Additionally, suspensions
of the active compounds may be prepared as appropriate oily
10   injection suspensions. Suitable lipophilic solvents or
vehicles include fatty oils such as sesame oil, or synthetic
fatty acid esters, such as ethyl oleate or triglycerides, or
liposomes. Aqueous injection suspensions may contain
substances which increase the viscosity of the suspension,
15   such as sodium carboxymethyl cellulose, sorbitol, or dextran.
Optionally, the suspension may also contain suitable
stabilizers or agents which increase the solubility of the
compounds to allow for the preparation of highly concentrated
solutions.
20   Alternatively, the active ingredient may be in powder
form for constitution with a suitable vehicle, e.g., sterile
pyrogen-free water, before use.

The compounds may also be formulated in rectal
compositions such as suppositories or retention enemas, e.g.,
25   containing conventional suppository bases such as cocoa
butter or other glycerides.

In addition to the formulations described previously,
the compounds may also be formulated as a depot preparation.
Such long acting formulations may be administered by
30   implantation (for example subcutaneously or intramuscularly)
or by intramuscular injection. Thus, for example, the
compounds may be formulated with suitable polymeric or
hydrophobic materials (for example as an emulsion in an
acceptable oil) or ion exchange resins, or as sparingly
35   soluble derivatives, for example, as a sparingly soluble
salt.

A pharmaceutical carrier for the hydrophobic molecules
of the invention is a cosolvent system comprising benzyl

- 28 -

alcohol, a nonpolar surfactant, a water-miscible organic
polymer, and an aqueous phase. The cosolvent system may be
the VPD co-solvent system. VPD is a solution of 3% w/v
benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate
80, and 65% w/v polyethylene glycol 300, made up to volume in
absolute ethanol. The VPD co-solvent system (VPD:5W)
consists of VPD diluted 1:1 with a 5% dextrose in water
solution. This co-solvent system dissolves hydrophobic
compounds well, and itself produces low toxicity upon
systemic administration. Naturally, the proportions of a co-
solvent system may be varied considerably without destroying
its solubility and toxicity characteristics. Furthermore,
the identity of the co-solvent components may be varied: for
example, other low-toxicity nonpolar surfactants may be used
instead of polysorbate 80; the fraction size of polyethylene
glycol may be varied; other biocompatible polymers may
replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and
other sugars or polysaccharides may substitute for dextrose.

     Alternatively, other delivery systems for hydrophobic
molecules may be employed. Liposomes and emulsions are well
known examples of delivery vehicles or carriers for
hydrophobic drugs. Certain organic solvents such as
dimethylsulfoxide also may be employed, although usually at
the cost of greater toxicity. Additionally, the compounds
may be delivered using a sustained-release system, such as
semipermeable matrices of solid hydrophobic polymers
containing the therapeutic agent. Various of sustained-
release materials have been established and are well known by
those skilled in the art. Sustained-release capsules may,
depending on their chemical nature, release the compounds for
a few weeks up to over 100 days. Depending on the chemical
nature and the biological stability of the therapeutic
reagent, additional strategies for protein stabilization may
be employed.

     The pharmaceutical compositions also may comprise
suitable solid or gel phase carriers or excipients. Examples
of such carriers or excipients include but are not limited to
calcium carbonate, calcium phosphate, various sugars,

starches, cellulose derivatives, gelatin, and polymers such
as polyethylene glycols.

7.4.3.   Effective Dosage.

5   Pharmaceutical compositions suitable for use in the
present invention include compositions wherein the active
ingredients are contained in an effective amount to achieve
its intended purpose. More specifically, a therapeutically
effective amount means an amount effective to prevent
10   development of or to alleviate the existing symptoms of the
subject being treated. Determination of the effective
amounts is well within the capability of those skilled in the
art, especially in light of the detailed disclosure provided
herein.

15   For any compound used in the method of the invention,
the therapeutically effective dose can be estimated initially
from cell culture assays. For example, a dose can be
formulated in animal models to achieve a circulating
concentration range that includes the IC_{50} as determined in
20   cell culture (i.e., the concentration of the test compound
which achieves a half-maximal CTGF activity). Such
information can be used to more accurately determine useful
doses in humans.

          A therapeutically effective dose refers to that amount
25   of the molecule hat results in amelioration of symptoms or a
prolongation of survival in a patient. Toxicity and
therapeutic efficacy of such molecules can be determined by
standard pharmaceutical procedures in cell cultures or
experimental animals, e.g., for determining the LD_{50} (the dose
30   lethal to 50% of the population) and the ED_{50} (the dose
therapeutically effective in 50% of the population). The
dose ratio between toxic and therapeutic effects is the
therapeutic index and it can be expressed as the ratio
between LD_{50} and ED_{50}. Molecules which exhibit high
35   therapeutic indices are preferred. The data obtained from
these cell culture assays and animal studies can be used in
formulating a range of dosage for use in human. The dosage
of such molecules lies preferably within a range of

circulating concentrations that include the ED_{50} with little
or no toxicity. The dosage may vary within this range
depending upon the dosage form employed and the route of
administration utilized. The exact formulation, route of
administration and dosage can be chosen by the individual
physician in view of the patient's condition. See, e.g.,
Fingl et al., 1975, in "The Pharmacological Basis of
Therapeutics", Ch. 1 p.1.

Dosage amount and interval may be adjusted individually
to provide plasma levels of the active moiety which are
sufficient to maintain the induction effects of CTGF, or
minimal effective concentration (MEC). The MEC will vary for
each compound but can be estimated from in vitro data; for
example, the concentration necessary to achieve 50-90%
activity of CTGF to induce bone growth using the assays
described herein. Dosages necessary to achieve the MEC will
depend on individual characteristics and route of
administration. However, HPLC assays or bioassays can be
used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value.
Compounds should be administered using a regimen which
maintains plasma levels above the MEC for 10-90% of the time,
preferably between 30-90% and most preferably between 50-90%.

In cases of local administration or selective uptake,
the effective local concentration of the drug may not be
related to plasma concentration.

The amount of composition administered will, of course,
be dependent on the subject being treated, on the subject's
weight, the severity of the affliction, the manner of
administration and the judgment of the prescribing physician.

7.4.4.   Packaging

The compositions may, if desired, be presented in a
pack or dispenser device which may contain one or more unit
dosage forms containing the active ingredient. The pack may,
for example, comprise metal or plastic foil, such as a
blister pack. The pack or dispenser device may be
accompanied by instructions for administration. Compositions

- 31 -

comprising a compound of the invention formulated in a
compatible pharmaceutical carrier may also be prepared,
placed in an appropriate container, and labelled for
treatment of an indicated condition. Suitable conditions
5   indicated on the label may include treatment of disorders or
diseases in which cartilage or bone induction and wound
healing, or the like is desired.

        7.5   Identification Of Compounds Which Induce The
10   Production of CTGF In Cartilage And

              The identification of the promoter element of the
CTGF gene and specifically, the TGF-β responsive/regulatory
element (TβRE) (5'-GTGTCAACCCTC-3'; nucleotides –157 and –
15   145), provides a source for a screening method for
identifying compounds or compositions which affect the
expression of CTGF. Specifically, the method by which
compositions which enhance the activity of CTGF, and thereby
may be used to enhance bone, tissue and cartilage induction
20   may be identified, comprises: (1) incubating components such
as, but not limited to, oligonucleotides comprising the
composition and a TGF-β responsive element of the CTGF
promoter, wherein said incubation is carried out under
conditions sufficient to allow the components to interact;
25   and (2) measuring the effect of the composition on CTGF
expression. Preferably, the promoter region used in the
screening assays described herein includes nucleotides –823
to +74, although smaller regions that include the TGF-β
responsive element may also be useful in the disclosed method
30   (e.g. –162 to –128, or –154 to –145). In other assays,
nucleotides in this region including TβRE are coupled to a
receptor gene such as luciferase and are transfected into
mammalian cells to derive a cell line, bearing a construct
and showing activity when incubated with TGF-β. These drugs,
35   oligonucleotides, and chemicals in libraries that modify the
activation can be readily detected in cell assays.

8.   EXAMPLES

The connective tissue growth factor gene is not
only expressed in fibroblasts but is selectively induced by
TGF-β only in mesenchymally derived connective tissue cells
5   (i.e., fibroblasts, smooth muscle, chondrocytes, osteoblasts,
astroglial cells, etc.). The expression of the gene
connective tissue cells that form the skeletal elements in
vertebrates indicate that CTGF plays a role in the formation
of cartilage, bone tendon and muscle in the vertebrate
10   animal. The results of the following examples demonstrate
that CTGF can regulate the induction, differentiation and
growth of cells which form both cartilage and bone in
vertebrate animals, including humans. Specifically, the
results provide that: (1) CTGF transcripts are present in the
15   growth plate of long bones in adult rats and newborn mice;
(2) The CTGF gene is expressed at sites of cartilage
induction and growth in embryonic mice; (3) CTGF receptors
are present on rat chondrocytes; (4) The CTGF gene is
expressed at site of bone regeneration after injury in adult
20   rabbits; (5) The CTGF protein can induce pluripotent mouse
embryonic stem cell lines to differentiate in to chondrocytes
and osteoblasts; 6) Human osteoblasts produce CTGF in
culture.

25   8.1 Biological Assays
*Methods: Mitogenic And Anchorage Independent
Growth Assays*. Mitogenic assays were performed in monolayer
cultures using 48 well plates and NRK fibroblasts as target
cells as described previously in Grotendorst, et al. 1991, *J.*
30   *Cell Physiol.* 149:235-243. Anchorage independent growth
assays were performed essentially as described in Guadagno
and Assoian, 1991, *J. Cell Biol.* 115:1572-1575.
Methods: Extracellular Matrix Protein mRNA
Induction Assays. NRK rat fibroblasts were grown to
35   confluence in Dulbecco's modified eagle media with 5% gfetal
bovine serum and then serum starved in DMEM with 1% bovine
serum albumin for 24 hours. Growth factors were added to the
cell cultures and total cellular RNA was extracted after 24

- 33 -

hours and northern blot analysis was performed as described
in Igarashi, et al., 1993, *Mol. Biol. Cell* 4:637-645.

5   To ensure that equivalent amounts of total RNA were
added to each lane on a gel, RNA was quantitated by $A_{260/280}$
ratios and equivalent transfer was assured by comparing
ribosomal 28S and 18S RNA bands in each lane after staining
with ethidium bromide. As additional control, blots were
reprobed with an actin cDNA probe. Double stranded cDNA
fragments used for probes were labeled with ^{32}P-dCTP using a
10   random prime labeling kit (Boehringer Mannheim, Indianapolis,
IN). The CTGF probe was derived from a 1.1 kb human cDNA
fragment which encompassed the open reading frame of the CTGF
transcript. The TGF-β1 probe was a 1.0 kb Nar I fragment
derived from a 2.0 kb human TGF-β1 cDNA (G.I. Bell, H.H.
15   Medical Institute, University of Chicago). The αI-type 1
human collagen probe was derived from a 1.5 kb ORF fragment
at the 3'end (ATCC No. 61323). The α5 integrin probe was
produced from a cDNA insert containing a portion of the human
cDNA containing the open reading frame, as obtained by R.
20   Associan at the University of Miami. The human fibronectin
probe was a 0.9 kb EcoR1/HindIII fragment derived from a 2.2
kb cDNA clone containing the 3' region of the open reading
frame provided by F. Woessner (also of University of Miami).
The human actin probe, used as the control RNA probe, was
25   purchased from Oncor, Co. (Gaithersberg, MD).

     8.2   Locus Of CTGF Transcripts In Newborn Mice
          Experiments were conducted to determine whether
CTGF transcripts are present in the growth plate of long
30   bones of newborn mice according to Fava, et al., 1990, *Blood*
76:1946-1955.
          Method: In situ Hybridization. The tissue samples
were immediately placed in 4.0% paraformaldehyde for 1.5
hours and then flash frozen and embedded. Sections were cut
35   at 5 μm and placed on TESPA coated slides (Oncor,
Gathersburg, MD). In-situ hybridization for CTGF mRNA was
performed using standard methods. Briefly, slides with
specimens were hydrated through graded alcohols, treated with

20 μg/ml proteinase K in 50 mM Tris-HCl ph 7.4, 5 mM EDTA,
refixed in 4.0% paraformaldehyde and dipped in 0.1 M
triethanolamine and 1 ml acetic anhydride, prior to
dehydration in sequentially graded alcohols. Both sense and
antisense CTGF RNA probes were constructed using a riboprobe
kit (Promega, Madison, WI) with T7 and Sp6 promoters,
respectively. The specific activity of the probes was 1 x
10^8 cpm/μg RNA. Slides were hybridized overnight in 50%
deionized formamide, 10% dextran sulfate, 50 mM DTT, 0.3M
NaCl, 0.01 M Tris pH 7.5, 5 mM EDTA, 10 mM Na2HPO4, 0.02 %
Ficoll, 0.02 % PVP, 0.02 % BSA, 0.2 mg/ml yeast tRNA and
the riboprobe (5 x 10^4 cpm/μl) under a coverslip at 54° C.
Slides were washed in 250 ml 5X SSC, 10 mM beta
mercaptoethanol at 50° C for 30 minutes, 2x SSC, 100mM beta
mercaptoethanol, 50% formamide at 65° C for 20 minutes and 3
times in TEN buffer (1 M Tris, 0.5 M EDTA, 5M NaCl) for 10
minutes. The second TEN wash included 10 μg RNase A. The
final two washes were in 2X SSC at 65° C. for 15 minutes
each. After dehydrating again through graded alcohols with
0.3 M ammonium acetate, the slides were dipped in
photographic emulsion (Ilford K-5, Polyscience) and incubated
for 8 days at 4° C. Slides were then developed and sections
counter stained in Mayer's hematoxylin and eosin.

       Results. The results of these studies indicate
that the CTGF gene is expressed in the proliferation zone of
the growth plate. This zone contains the chrondrocytes that
are actively replicating to increase the length of the bone.
The expression of CTGF at this site is consistent with it
functioning as a growth factor for the chondrocytes.

      **8.3 CTGF Gene Expression At Site Of Cartilage Induction
           And Growth In Embryonic Mice.**

       In order to confirm CTGFs role in cartilage
induction and growth, the expression of the CTGF gene in
mouse embryo's at sites where cartilage and bone will form
but have not formed yet was studied. For purposes of this
study, a transgenic mouse line which contains a transgene
composed of the human CTGF promoter elements which are

regulating the expression of the bacterial β-galactosidase
gene. Cells that express this gene can be readily identified
by staining using X-gal which forms a blue colored
precipitate at the sites of enzymatic activity. Using this
5   methodology we stained embryo's from transgenic mice to
localize the expression of the CTGF gene. As set forth at
FIGURE 4, Panel A, no cartilage or bone was formed,
indicating the CTGF is expressed prior to the formation of
the skeleton and could function as the inducer of cartilage
10 and bone. These results further demonstrate that the
expression of the transgene corresponds with the expression
detected by in situ hybridization using the CTGF probe.

       These studies also demonstrate that the gene is
expressed at growth plates in the long bone, in
15 precartilaginous zones and in Meckel's cartilage, the first
cartilage to form during mammalian development. These areas
are referred to as prechondrogenic mesenchyme and are
distinguished by condensations of the cells. The CTGF gene
is expressed in these sites but not in adjacent tissue.
20 Furthermore, the CTGF gene is expressed at these sites 1 day
prior to the condensation which occurs 1 day prior to the
actual formation of the cartilage. These findings
demonstrate that CTGF is present prior to the formation of
cartilage or cells with a true chondrocytic phenotype, and is
25 consistent with CTGF acting to induce the cartilage phenotype
in undifferentiated stem cells.

       Importantly, these studies demonstrate that CTGF is
expressed at sites in the embryo that form bone by either the
intramembranous, or endochondral pathways, demonstrating that
30 it can function as a signal for cartilage development from
either non-differentiated mesenchymal stem cells which form
the bones of the limbs, or neural crest cells which form the
cartilage in Meckel's cartilage and the bones of the skull.

35   8.4 Loci Of CTGF Receptors On Rat Chondrocytes
           In order for cells to respond to peptide factors
       such as CTGF, they must express on their surface the cognate
       receptor for the specific peptide factor.

Equilibrium Assay. Equilibrium binding assays were
performed on confluent monolayers of NRK-49F rat fibroblasts
and primary rat articular chondroblasts to determine the
number and affinity of CTGF receptors on these cells.
5   Binding was performed in the cold for 4 hours with varying
concentrations of iodinated recombinant human CTGF (rhCTGF).
Non-specific binding was determined by including a 200-fold
molar excess of unlabeled ligand. Representative Scatchard
plots are set forth at FIGURE 8A (with respect to equilibrium
10   binding assays perfomed using NRK cells) and FIGURE 8B (with
respect to equilibrium binding assays performed using rat
chondroblasts).

Competition Assay. Several cell types were tested
for the expression of CTGF receptors including, normal rat
15   kidney fibroblasts, mouse fibroblasts, mink lung epithelial
cells and rat articular chondrocytes. CTGF was labeled by
iodination with ^{125}I and the radiolabled CTGF used in
competition binding assays to measure CTGF receptors on the
various cell types. As set forth in Table 1, below, only the
20   NRK fibroblasts and rat articular chondrocytes expressed high
affinity receptors for CTGF. Mouse fibroblasts had few if
any high affinity receptors and no binding was detected in
the mink lung epithelial cells.

25   TABLE 1
         BINDING CHARACTERISTIC FOR rhCTGF ON VARIOUS CELLS

Cell Type	High Affinity		Low Affinity	
	KD (pM)	Sites/cell	KD (nM)	Sites/cell
NRK	13-23	2200-3500	1.1-2.2	126,000-195,000
Chondrocytes	21	3500-4800	1.0	150,000
NIH3T3	5-10	480	1.8	102,000
MLEC	none detected		none detected	

These data indicate that chondrocytes express both CTGF
and its receptor and would are therefore capable of
responding to CTGF as a growth stimulatory factor.

                              - 37 -

8.5 **CTGF Activity In Inducing Pluripotent Mouse
      Embryonic Stem Cell Lines To Differentiate Into
      Chondrocytes And Osteoblasts.**

The ability of CTGF to induce the chondrocytic and
osteocytic phenotype in undifferentiated stem cells in cell
culture was evaluated. Specifically, the cell line C3H10T1/2
was used to evaluate this biological activity. These cells
are a standard and well established line for these types of
investigations. The C3H10T1/2 can be maintained in an
undifferentiated state in culture, and then induced to
differentiate into skeletal muscle cells, chondrocytes,
osteoblasts and adipocytes. Cells treated with CTGF formed
colonies of chondocytes and cartilaginous nodules. Cells
treated overnight with 5-azacytodine overnight followed by
treatment with CTGF differentiated into osteoblasts and
osteoid bodies. The differentiation of these cultures into
muscle and adipocytes was blocked by the presence of CTGF.

More specifically, the cells were treated overnight with
5 azacytodine followed by a 10-14 day incubation to allow for
the differentiation to occur. The effects of 5 azacytodine
and CTGF alone and in combination on these cell were then
compared. Control cultures which were not treated with
either agent remained as undifferentiated cells in monolayer.
As set forth as FIGURE 4, cultures treated with 5azacytodine
alone differentiated into primarily skeletal muscle cells
(myotubes) and adipocytes. No chondrocytes could be found in
the cultures. CTGF treatment of the cultures (50 ng/ml) for
10 days resulted in the induction of cartilaginous nodules.
These nodules were not found under any other conditions.
Treatment of the cultures with FGF, PDGF, EGF or TGF-β did
not induce these nodules indicating that CTGF is uniquely
capable of inducing cartilage in the undifferentiated
mesenchymal stem cells.

Treatment of the cells with both 5 azacytodine
(overnight) followed by a 10 day exposure to CTGF (50 ng/ml)
had a significant effect on the cultures. First, no skeletal
myotubes where present demonstrating that CTGF prevented the
cells from differentiating into skeletal muscle cells.

- 38 -

Second, while some cartilaginous nodules were present most of the nodules appeared to be osteoid (bone). Thus, CTGF can induce the formation of both chondrocytes and osteoblasts from undifferentiated mesenchymal cells. These results demonstrate that this factor could be used to stimulate the differentiation of cartilage and bone where desired.

8.6 CTGF Gene Expression At Site Of Bone Regeneration After Injury In Adult Rabbits.

An experimental model was developed to examine the expression of various regulatory and matrix protein genes during wound repair. In this model, mesh nylon cylinders are implanted in the ilium of the pelvis of male New Zealand white rabbits (10 kg) which had been anesthetized by ether. A 1.1 cm diameter hole was bored in the ilium of the pelvis using a bone trephine and the chamber press fit into the hole. The chamber was anchored in place using flaps of the adjacent musculature and ligaments. Two chambers were implanted in each of twenty animals.

Animals were sacrificed on Days 9, 14, 21, 24, 28, 31, 35, 42 and 56 after implantation of the chambers. The chambers were removed and the tissue on the outside of the chambers were carefully and completely removed. The chambers were then cut open and tissue contained within the chambers was collected.

Total RNA was extracted from the tissue obtained from 6-18 chambers (pooled from 1-3 animals) by Guanidine-isothiocynate extraction (Chomcaynski and Sacchi, 1987, *Anal. Biochem.* 162:156-159) and CsCl centrifugation (Chirgwin, et al., 1979, *Biochemistry* 18:5294-5299). The amount of RNA recovered ranged from 100-300µg during the different days of collection. Total RNA was electrophoresed on an agarose/formaldehyde gel and transferred to nitrocellulose. Equivalent amounts of RNA were transferred as judged from staining of the ribosomal RNA present in each sample on the nitrocellulose filter. The CTGF probe was a 900 base pair fragment which represented the open reading frame of the CTGF cDNA. Hybridizations were performed using 1×10^6 cpm/ml of

these probes labeled with [P³²]dCTP by using a Random Primer
DNA Labeling Kit (Boehringer Mannheim Biochemicals,
Indianapolis, IN). Autoradiography was performed at -70°C
for 24-72 hours by using X-ray films and intensifying
screens.

     The tissue proceeded through a regular cascade of repair
where blood coagulation is followed by inflammation and then
connective tissue in growth. In the bone implanted chambers
the dense connective tissue which formed was similar if not
indistinguishable from that which forms in soft tissue
implants.

     As set forth in FIGURE 5, CTGF gene expression is
evident from Days 14-42. This is 4 days prior to the first
histological appearance of bone within the chambers and
coincides with the time course for the formation of bone
within the chambers. However, as also set forth at FIGURE 5,
around day 17-18 post-implantation there were some changes in
the morphology of areas of the connective tissue. These
areas then began to form bone by day 20-21 post-implantation,
demonstrating that this is a functional model for the study
of bone regeneration.

     The expression of CTGF mRNA in the chambers preceded
slightly and then coincided with the formation and growth of
the osteogenic areas within the chamber, demonstrating that
CTGF is expressed at sites of bone regeneration in mammals.

     8.7 **Human Osteoblast Formation By Administration Of
          CTGF**

          Cell culture. Human osteoblasts were grown from
explants of human bone. Cells were cultured in Dulbecco's
modified Eagle's medium (DMEM) containing 10 % fetal calf
serum (FCS) at 37⁻C in an atmosphere of 10 % CO_2 and 90 %
air.

          Western blot analysis. CTGF content in conditioned
media was analyzed by SDS-PAGE on 12 % acrylamide gels
followed by transfer to nitrocellulose filters using
electroblotting. The blots were incubated for 1 hour in
Tris-buffered saline (100mM NaCl, 50mM Tris-HCl pH7.4) with 2

- 40 -

% nonfat powdered milk (TBS-milk), prior to overnight
exposure to 2 g/ml chicken anti-human CTGF IgY diluted in
TBS-milk. Filters were washed five times in TBS-milk, 5
minutes each, and incubated with alkaline-phosphatase-
conjugated affinity purified rabbit anti-chicken IgY (1:1,000
dilution, Organon Teknika-Cappel, West Chester, Pa.) in TBS-
milk for 90 minutes. The filters were washed three times
with TBS-milk followed by two washes in TBS, and the antigens
were detected using a commercial alkaline phosphatase
substrate kit (Sigma, St. Louis, MO).

        Results. Human osteoblasts were obtained from
donors after surgical removal of bone during procedures to
remove bone tumors or joint replacement. Osteoblasts were
cultured from the bone and identified using standard. Cells
were grown to confluence in complete media containing 10 %
fetal calf serum and made quiescent by changing the media to
serum free media overnight. Some cultures were treated with
TGF-β and compared to non-treated cultures. The osteoblast
that were treated with TGF-β were stimulated to produce CTGF,
as detected with a specific anti-CTGF antibody. As set forth
at FIGURE 5, the media was collected and analyzed for CTGF
production and secretion by immunopurification of the CTGF
with a CTGF specific antibody and detection and quantitation
by western blots using the same antibody. As observed with
fibroblasts, smooth muscle cells, and chondrocytes, TGF-β
induces CTGF production by the human osteoblasts. Control
non-treated cells did not synthesis detectable amounts of
CTGF.

        As evidenced by this experiment, osteoblasts respond to
TGF-β similarly to other connective tissue cells with regard
to CTGF production.

8.8 Transgenic Rabbit Models

        All mice studies were conducted in accordance with
the principles and procedures outlined in "Guidelines for
Care and Use of Experimental Animals". Generation of
transgenic models was carried out at the University of Miami
Transgenic Mouse Core Facility using standard techniques.

Briefly, the gene to be injected (transgene) was linearized
by restriction digestion and the DNA fragment isolated by low
melt agarose gel electrophoresis and purified using
GENECLEAN.

5   Transgenic mice were generated by injecting linearized
DNA into one of the pronuclei of ~100-300 recently fertilized
mouse ova. Hogan, et al.., 1986, Manipulating the Mouse
Embryo, Cold Spring Harbor Laboratory Press, Cold Spring
Harbor, NY. Those eggs that survive injection were

10   transferred to the oviducts of pseudopregnant mice (mated to
vasectomized males). One to three weeks after birth a tail
biopsy will be taken from the pups and genomic DNA analyzed
by southern blot to determine the presence of the transgene.
Mice that were positive for the presence of the transgene

15   were mated to control mice to establish transgenic mouse
lines. As a result of these experiments, two independent
lines of transgenic mice that express the β-galactosidase
under CTGF promoter control were produced. Both of these
lines exhibit similar patterns of expression.

20

        8.9   Chondrogenic Assay
             CTGF, as well as TGF-β were tested in a
chondrogenic assay as described in Seydin, et al., 1983, J.
Cell Biology 97:1950-53. Briefly, primary cultures of

25   embryonic muscle were obtained from cellular outgrowth of
minced muscle tissue dissected from limbs of 19-20 day old
Sprague-Dawley fetuses. For the chondrogenic assay, the
cells were trypsinized and embedded in agarose, and overlayed
with media containing no factors (FIGURE 7A), TGF-β alone

30   (FIGURE 7B), TGF-β and cholera toxin (FIGURE 7C) or TGF-β,
cholera toxin and CTGF (Figure 7D). For each assay, media
were changed every 2 - 3 days and after 21 days of culture
stained with Toluidine blue as described in Horwitz and
Dorfman, 1970, J. Cell Biol. 45:434-438.

35   As set forth in FIGURES 7A-7D, marked chondrocyte growth
was observed where CTGF was added to media, indicating that
CTGF stimulates chondrocyte growth, and the production of
connective tissue matrix.

                              - 42 -

The present invention is not to be limited in scope by
the exemplified embodiments which are intended as
illustrations of single aspects of the invention, and methods
which are functionally equivalent are within the scope of the
5   invention. Indeed, various modifications of the invention in
addition to those described herein will become apparent to
those skilled in the art from the foregoing description and
accompanying drawings. Such modifications are intended to
fall within the scope of the appended claims.

10   All references cited within the body of the instant
specification are hereby incorporated by reference in their
entirety.

Biological Deposits

The sequence of the CTGF of the invention was
15   deposited with Genebank, Los Alamos National Laboratory, Los
Alamos, New Mexico, 87545, USA, on July 26, 1990, and given
an accession no. M36965. The deposit of this CTFG sequence
is for exemplary purposes only, and should not be taken as an
admission by the Applicant that such deposit is necessary for
20   enablement of the claimed subject matter.

In respect of all designated States in which such
action is possible and to the extent that it is legally
permissible under the law of the designated State, it is
requested that a sample of the deposited micro-organism be
25   made available only by the issue thereof to an independent
expert, in accordance with the relevant patent legislation,
e.g., EPC rule 28(4), United Kingdom Patent Rules 1982 rule
17(3), Australian Regulation 3.25(3) and generally similar
provisions *mutatis mutandis* for any other designated State.

30

International Application No: PCT/   /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 43, lines 1-25 of the description '

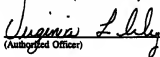**A. IDENTIFICATION OF DEPOSIT '**

Further deposits are identified on an additional sheet '

Name of depositary institution '

GeneBank Submissions

Address of depositary institution (including postal code and country) '

Los Alamos National Laboratory
Los Alamos, NM 87545
US

Date of deposit ' July 26, 1990 Accession Number ' M36965

B. ADDITIONAL INDICATIONS ' (leave blank if not applicable). This information is continued on a separate attached sheet

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C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ' (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS ' (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later ' (Specify the general nature of the indications e.g.,
"Accession Number of Deposit")

E. ☒ This sheet was received with the International application when filed (to be checked by the receiving Office)

Virginia L. Lily
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau "

was _____
                                                    (Authorized Officer)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED:

1.   A pharmaceutical composition comprising CTGF.

2.   A method for inducing bone formation comprising the administration to a patient in need a composition comprising CTGF and a pharmaceutical acceptable carrier.

3.   The method of claim 2 wherein said composition further comprises a second growth factor.

4.   The method of claim 3 wherein the second growth factor is TGF-β.

5.   The method of claim 2 wherein said composition is further comprised of at least one collagen.

6.   The method of claim 2 wherein the patient is suffering from an affliction which affects bone formation.

7.   The method of claim 6 wherein the affliction is selected from the group consisting of osteoporosis, osteoarthritis and osteochondrytis.

8.   A method for inducing tissue formation comprising the administration to a patient in need a composition comprising CTGF and a pharmaceutical acceptable carrier.

9.   The method of claim 8 wherein said composition further comprises a second growth factor.

10.   The method of claim 8 wherein the second growth factor is TGF-β.

11.   The method of claim 8 wherein said composition is further comprised of at least one collagen.

12.   A method for inducing cartilage formation comprising the administration to a patient in need a composition comprising CTGF and a pharmaceutical acceptable carrier.

13.   The method of claim 12 wherein said composition further comprises a second growth factor.

14.   The method of claim 13 wherein the second growth factor is TGF-β.

15.   The method of claim 12 wherein said composition is further comprised of at least one collagen.

16.   A method for inducing wound healing comprising the administration to a patient in need a composition comprising CTGF and a pharmaceutical acceptable carrier.

17.   The method of claim 16 wherein said composition further comprises a second growth factor.

18.   The method of claim 16 wherein the second growth factor is TGF-β.

19.   The method of claim 16 wherein said composition is further comprised of at least one collagen.

PCT/US 96/08210

FIGURE 1

Nucleotide sequence of the CTGF gene

```
-823  TCTAGAGCTCGCGCGAGCTCTAATACGACTCACTATAGGGCGTCGACTCGATCCCTTTTT

-763  CTGGAAACATTGATGGCCACTCGTCCCTTGTCCTTGCCTATATAAAACTCCTACATATAT

-703  TAAGAGAAAACTAAGCAAGAGTTTTGGAAATCTGCCCCAGGAGACTGCATCCTGAGTCAC
                                                            AP-1
-643  ACGAGTCTTTGTTCTCTTTCTTGTCCCAAAACCGTTACCTCAAGTGACAAATGATCAAAT

-583  CTCAAATATAGAATTCAGGGTTTTACAGGTAGGCATCTTGAGGATTTCAAATGGTTAAAA

-523  GCAACTCACTCCTTTTCTACTCTTTGGAGAGTTTCAAGAGCCTATAGCCTCTAAAACGCA

-463  AATCATTGCTAAGGGTTGGGGGGGAGAAACCTTTTCGAATTTTTTAGGAATTCCTGCTGT

-403  TTGCCTCTTCAGCTACCTACTTCCTAAAAAGGATGTATGTCAGTGGACAGAACAGGGCAA
                           CArG box
-343  ACTTATTCGAAAAAGAAATAAGAAATAATTGCCAGTGTGTTTATAAATGATATGAATCAG
                                                         AP-1
-283  GAGTGGTGCGAAGAGGATAGGAAAAAAAAAATTCTATTTGGTGCTGGAAATACTGCGCTTT

-223  TTTTTTCCTTTTTTTTTTTTTTTTCTGTGAGCTGGAGTGTGCCAGCTTTTTCAGACGGAGGAA
                                        NF-1 like
-163  TGCTGAGTGTCAAGGGGTCAGGATCAATCCGGTGTGAGTTGATGAGGCAGGAAGGTGGGG
                                             TIE-like
-103  AGGAATGCGAGGAATGTCCCTGTTTGTGTAGGACTCCATTCAGCTCATTGGCGAGCCGCG
                                                       CATbox
 -43  GCCGCCCGGAGCGTATAAAAGCCTCGGGCCGCCCGCCCCAAACTCACACAACAACTCTTC
        SP-1           TATAbox         SP-1         +1
  17  CCCGCTGAGAGGAGACAGCCAGTGCGACTCCACCCTCCAGCTCGACGGCAGCCGCCCCGG

  77  CCGACAGCCCCGAGACGACAGCCCGGCGCGTCCCGGTCCCCACCTCCGACCACCGCCAGC

 137  GCTCCAGGCCCCGCGCTCCCCGCTCGCCGCCACCGCGCCCTCCGCTCCGCCCGCAGTGCC

 197  AACCATGACCGCCGCCAGTATGGGCCCCGTCCGCGTCGCCTTCGTGGTCCTCCTCGCCCT
         M  T  A  A  S  M  G  P  V  R  V  A  F  V  V  L  L  A
 257  CTGCAGCCGGGTAAGCGCCGGGAGCCCCCGCTGCGGCCCGGCGGCTGCCAGGGAGGGACTC
      L  C  S  R .......... Intron 1 ......................
 317  GGGGCCGGCCGGGGAGGGCGTGCGGCGCCGACCGAGCGCCGCTGACCGCCCTGTCCTCCCT
 377  GCAGCCGGCCGGTCGGCCAGAACTGCAGCGGGCCGTGCCGGTGCCCGGACGAGCCGGCGCC
      .... P  A  V  G  Q  N  C  S  G  P  C  R  C  P  D  E  P  A
 437  GCGCTGCCCGGCGGGCGTGAGCCTCGTGCTGGACGGCTGCGGCTGCTGCCGCGTCTGCGC
      P  R  C  P  A  G  V  S  L  V  L  D  G  C  G  C  C  R  V  C
 497  CAAGCAGCTGGGCGAGCTGTGCACCGAGCGCGACCCCTGCGACCCGCACAAGGGCTCTT
      A  K  Q  L  G  E  L  C  T  E  R  D  P  C  D  P  H  K  G  L
 557  CTGTGACTTCGGCTCCCCGGCCAACCGCAAGATCGGCGTGTGCACCGGTAAGACCCGCAG
      F  C  D  F  G  S  P  A  N  R  K  I  G  V  C  T ...Intron 2..
```

FIGURE 1C.1

```
 617 CCCCCACCGCTAGGTGTCCGGCCGCCTCCTCCCTCACGCCCACCCGCCCGCTGGAAAAAG
 677 AAACCGCTCGGACTGAGTTTCTTTCTCCAGCTGCTGCCAGCCCGCCCCTGCAGCCCAGA
 737 TCCCAACTCGCATCCCTGACGCTCTGGATGTGAGAGTGCCCCAATGCCTGACCTCTGCAT
 797 CCCCCACCCCTCTCTTCCCTTCCTCTTCTCCAGCCAAAGATGGTGGTCCCTGCATCTTCG
                           .......... A  K  D  G  A  P  .C  I  F
 857 GTGGTACGGTGTACCGCAGCGGAGAGTCCTTCCAGAGCAGCTGCAAGTACCAGTGCACGT
       G  G  T  V  Y  R  S  G  E  S  F  Q  S  S  C  K  Y  Q  C  T
 917 GCCTGGACGGGGCGGTGGGCTGCATGCCCCTGTGCAGCATGGACGTTCGTCTGCCCAGCC
       C  L  D  G  A  V  G  C  M  P  L  C  S  M  D  V  R  L  P  S
 977 CTGACTGCCCCTTCCCGAGGAGGGTCAAGCTGCCCGGGAAATGCTGCGAGGAGTGGGTGT
       P  D  C  P  F  P  R  R  V  K  L  P  G  K  C  C  E  E  W  V
1037 GTGACGAGCCCAAGGACCAAACCGTGGTTGGGCCTGCCCTCGCGGGTGAGTCGAGTCTTC
       C  D  E  P  K  D  Q  T  V  V  G  P  A  L  A  ..............
1097 CTCTAAGTCAGGGTCGTGATTCTCTCCCAGGGAGGGAGTCCTAACTGTGCCGACCGAACG
       ..........Intron 3 ..................................
1157 GGGAAATACCTTATCAGGCGTTTTACATGGTGTTTGTGTGTCTGCTCTGCAGCTTACC
                                                         A  Y
1217 GACTGGAAGACACGTTTGGCCCAGACCCAACTATGATTAGAGCCAACTGCCTGGTCCAGA
       R  L  E  D  T  F  G  P  D  P  T  M  I  R  A  N  C  L  V  Q
1277 CCACAGAGTGGAGCGCCTGTTCCAAGACCTGTGGGATGGGCATCTCCACCCGGGTTACCA
       T  T  E  W  S  A  C  S  K  T  C  G  M  G  I  S  T  R  V  T
1337 ATGACAACGCCTCCTGCAGGCTAGAGAAGCAGACCCGCCTGTGCATGGTCAGGCCTTGCG
       N  .D  N  A  S  C  R  L  E  K  Q  S  R  L  C  M  V  R  P  C
1397 AAGCTGACCTGGAAGAGAACATTAAGGTACATGTTCTGCTCCTATTAACTATTTTTCACA
       E  A  D  L  E  E  N  I  K ...........Intron 4 ...........
1457 GGAAAAACAGTGGATAGGACCCAACTTAGGGCTCTTGCACGCTTGTTAGTATAAGCCCGT
1517 TATCTCCAAAACTATCTAACCATTGAGCTGTTTTGCTGGAATGAGAGCTTGTGTAATAGC
1577 AACCACCAGTTTTCCACTACGAAATCTTCCACAGGGTTAGTTAATTCAAGACATTCCAAG
1637 AGAGGCTCTGGCTATTTTTGGACATAGCAAATGAGACTCAAACTTCCTCCCCCTCAAAATA
1697 TAAACAGAAGTCAGACAACAGAAGACTAAAACACAGAGGGTTGAAGAAAGCCACTCCTCT
1757 TGTAGAGTCGCTGATTTTTTTTTTCCTCTCTCTTTTCCCTTGTCTTCCTTAGAAGGGCA
                                                            K  G
1817 AAAAGTGCATCCGTACTCCCCAAAATCTCCAAGCCTATCAAGTTTGAGCTTTCTGGCTGCA
       K  K  C  I  R  T  P  K  I  S  K  P  I  K  F  E  L  S  G  C
1877 CCAGCATGAAGACATACCGAGCTAAATTCTGTGGAGTATGTACCGACGGGCCGATGCTGCA
       T  S  M  K  T  Y  R  A  K  F  C  G  V  C  T  D  G  R  C
1937 CCCCCCACAGAACCACCACCCTGCCGGTGGAGTTCAAGTGCCCTGACGGCGAGGTCATGA
       T  P  H  R  T  T  T  L  P  V  E  F  K  C  P  D  G  E  V  M*
1997 AGAAGAACATGATGTTCATCAAGACCTGTGCCTGCCATTACAACTGTCCCGGAGACAATG
       K  K  N  M  M  F  I  K  T  C  A  C  H  Y  N  C  P  G  D  N
2057 ACATCTTTGAATCGCTGTACTACAGGAAGATGTACGGAGACATGGCATGAAGCCAGAGAG
       D  I  F  E  S  L  Y  Y  R  K  M  Y  G  D  M  A  *
2117 TGAGAGACATTAACTCATTAGACTGGAACTTGAACTGATTCACATCTCATTTTTCCGTAA
2177 AAATGATTTCAGTAGCACAAGTTATTTAAATCTGTTTTCTAACTGGGGGAAAAGATTCC
2237 CACCCAATTCAAAACATTGTGCCATGTCAAACAAATAGTCTATCTTCCCCAGACACTGGT
2297 TTGAAGAATGTTAAGACTTGACAGTGGACTACATTAGTACACAGCACCAGAATGTGTATAT
2357 TAAGGTGTGGCTTTAGGAGCAGTGGGAGGGTACCGGCCCGGTTAGTATCATCAGATCGAC
2417 TCTTATACGAGTAATATGCCTGCTATTTGAAGTGTAATTGAGAAGGAAAATTTTAGCGTG
2477 CTCACTGACCTGCCTGTAGCCCCAGTGACAGCTAGGATGTGCATTCTCCAGCCATCAAGA
2537 GACTGAGTCAAGTTGTTCCTTAAGTCAGAACAGCAGACTCAGCTCTGACATTCTGATTCG
2597 AATGACACTGTTCAGGAATCGGAATCCTGTCGATTAGACTGGACAGCTTGTGGCAAGTGA
2657 ATTTGCCTGTAACAAGCCAGATTTTTTAAAATTTATATTGTAAATATTGTGTGTGTGT
2717 GTGTGTGTATATATATATATATATGTACAGTTATCTAAGTTAATTTAAAGTTGTTTGTGC
2777 CTTTTTATTTTTGTTTTTAATGCTTTGATATTTCAATGTTAGCCTCAATTTCTGAACACC
2837 ATAGGTAGAATGTAAAGCTTGTCTGATCGTTCAAAGCATGAAATGGATACTTATATGGAA
```

FIGURE 10 2

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2897 ATTCTGCTCAGATAGAATGACAGTCCGTCAAAACAGATTGTTTGCAAAGGGGAGGCATCA
2957 GTGTCTTGGCAGGCTGATTTCTAGGTAGGAAATGTGGTAGCTCACGTTTAATGAACAAAT
3017 GGCCTTATTAAAAACTGAGTGACTCTATATAGCTGATCAGTTTTTCACCTGAAGCATTTG
3077 TTTCTACTTTGATATGACTGTTTTTCGACAGTTTATTTGTTGAGAGTGTGACCAAAAGTT
3137 ACATGTTTGCACCTTTCTAGTTGAAAATAAAGTGTATATTTTTTCTATAAAGGGCTTGGT
3197 TATTCATTTATCCTTCTAAACATTTCTGAGTTTTCTTGAGCATAAATAGGAAGTTCTTAT
3257 TAATCATAAGATAATTCACCAATAATTTTCTAAATATCTTTAATTATTCTATACATTAAT
3317 AAATTGATTATTCCATAGAATTTTTATGTAAACATACTTCACACTGAATCAAGTATCACA
3377 GACTTGCAGGCATA
```

Figure 7. In situ hybridization detection of CTGF expression in
humerus of newborn mouse.

Figure 3. β-galactosidase expression in transgenic mice possesing the
CTGF promoter-β-galactosidase reporter fusion gene.

Panel A. Whole mount Day 12 mouse embryo.

Panel B.   Hind foot of newborn.

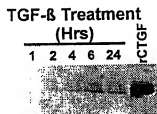Figure 4.   Effect of CTGF on the differentiation of C3H10T1/2 cells.
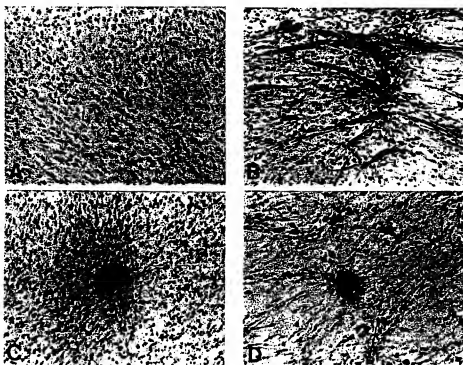
Figure 5. Northern blot of CTGF mRNA demonstrating the expression of the CTGF gene during bone repair.

DAY AFTER SURGERY

9 14 21 24 28 31 35 42 56

CTGF mRNA →
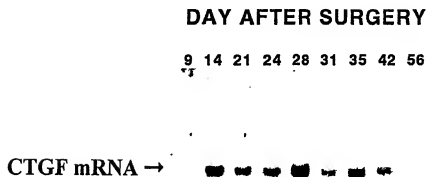
Figure 6. Induction of CTGF by TGF-β in human osteoblasts.
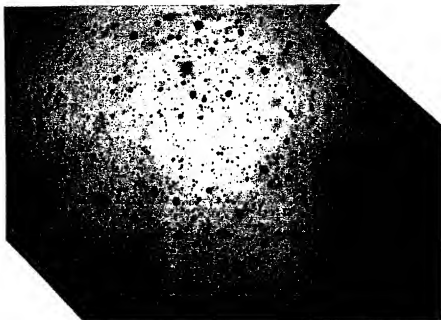
TGF-ß Treatment (Hrs)

1 2 4 6 24
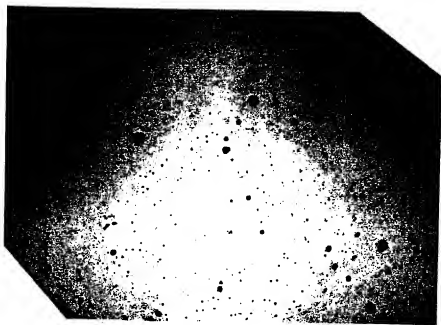
FIG 7A
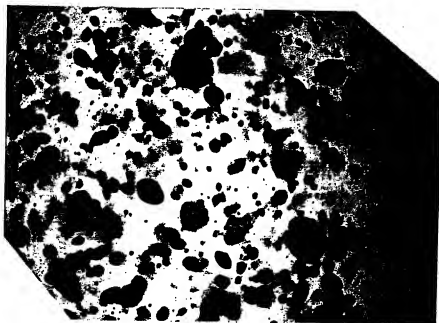
FIG 7B

FIG 7C
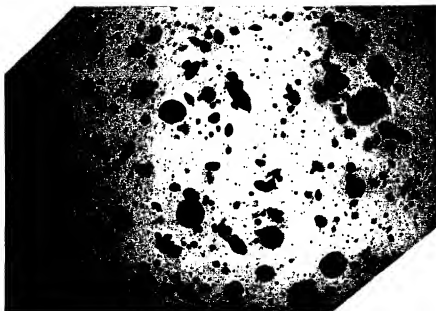
FIG 7D

FIGURE 8A

Scatchard Plot of CTGF Binding to NRK Cells

Bound
[fmoles per million cells]
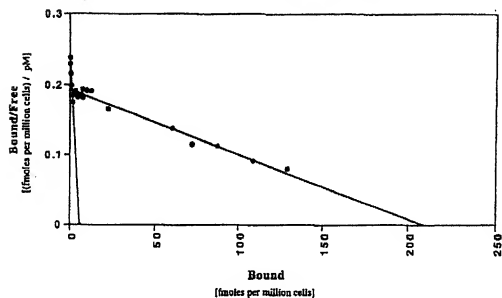
Scatchard Plot of CTGF Binding to Rat Chondroblasts

Bound
(fmoles per million cells)
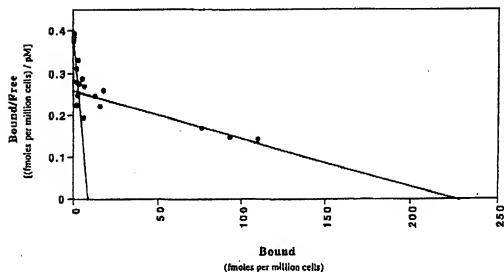
FIGURE 8B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08210

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/18, 38/39; C07K 14/495, 14/475, 14/78
US CL :424/198.1, 423, 484, 492; 530/350, 356

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/198.1, 423, 484, 492; 530/350, 356

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
search terms: connective tissue growth factor, TGF-beta, collagen, wound, bone, cartilage

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,408,040 (GROTENDORST ET AL.) 18 April 1995, see entire document, especially column 2, column 5.	1, 8-10, 16-18 ---------- 2-7, 11-15, 19
Y	US, A, 5,149,691(RUTHERFORD) 22 September 1992, see the claims.	2-7, 11-15, 19
Y	US, A, 5,356,630 (LAURENCIN ET AL.) 18 October 1994, see claim 1.	2-7, 11-15, 19
Y	US, A, 5,399,361 (SONG ET AL.) 21 March 1995, see column 2.	2-7, 11-15, 19

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

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"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 JULY 1996	22 JUL 1996

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